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Geographical Variability in Antibacterial Chemical Defenses of the Caribbean Sponge Xestospongia muta

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Geographical Variability in Antibacterial Chemical Defenses of the Caribbean Sponge *Xestospongia muta*

by
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Abstract

Coral reefs are important and diverse marine ecosystems, and sponges represent the majority of species in Caribbean reefs. *Xestospongia muta* is a giant barrel sponge that is one of the most abundant and visible sponges on Caribbean reefs, and has been the subject of many studies. *Xestospongia muta* produces a diversity of secondary metabolites that have various ecological functions including allelopathy, anti-fouling, predation deterrence, and antibacterial activity. The chemical defenses produced by a sponge can change in response to various biotic and abiotic stressors, including water temperature, sedimentation, nutrient runoff, and predation.

This study compared the antibacterial activity of *X. muta* samples from sites at four countries in the Caribbean: Belize, Curaçao, Grand Cayman, and St. Croix, US Virgin Islands, to characterize geographic variation in the bioactivity of secondary metabolites from *X. muta*. There are currently no known sponge pathogens of *X. muta*, so antibacterial activity was tested against three known coral pathogens, and one human enteric bacteria that can be discharged onto reefs. Antibacterial assays compared the growth of the bacterial strains exposed to sponge extracts with bacterial growth in control broth.

The sponges from each country had different antibacterial activity profiles, with sponges from Belize having the most inhibitory interactions and sponges from St. Croix having the least. The differences in activity between countries suggest that large-scale geographic patterns in metabolite production do occur. Within countries, the antibacterial

activity was largely the same, but there were some examples of significant differences. Many of the factors that can affect secondary metabolite production were similar except for the amount of human activity in the area, suggesting that anthropogenic factors can cause large changes in reef ecosystems. The sponges also varied in the amount of activity against each bacteria, suggesting that there is specificity in the action of the chemical defenses of *X. muta*.

As sponges continue to increase in abundance and biomass on Caribbean reefs, they will be exposed to greater impacts from natural and anthropogenic changes. Data from this study indicate that such changes could have important ecological implications on the ability of *X. muta* to produce chemical defenses to protect itself from potential pathogens and other stressors.

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Introduction

Sponge Ecology in Reef Ecosystems

In coral reef ecosystems of the Caribbean, sponges provide a majority of the biomass and species biodiversity (Diaz & Rutzler 2001). In addition to being some of the most abundant organisms, sponges perform multiple functional roles in reefs. Sponges can alter the carbonate structures of reefs by stabilizing sediments or eroding calcium carbonate skeletons left by corals (Diaz & Rutzler 2001; Bell 2008). Sponges provide habitats for symbiotic microorganisms (Diaz & Rutzler 2001), which can produce nutrients for the sponge and participate in nitrification of dissolved inorganic nitrogen (Diaz & Ward 1997). Sponges are filter feeding organisms, which enable them to cycle nutrients through the water column, coupling the reef areas at various depths (Lesser 2006; Bell 2008). Some sponges also serve as food sources and are preyed upon by various fish and other species (Loh & Pawlik 2014).

Since sponges are sessile organisms, they are not able to move to optimal locations and must adapt to local conditions wherever their larvae are able to settle and survive. As filter feeders, a sponge's diet consists primarily of carbon in two forms, particulate organic carbon (POC) and dissolved organic carbon (DOC) (Yahel et al. 2003, McMurray et al. 2016). POC is comprised mainly of bacteria, plankton, and other microorganisms, as well as detritus from dead organisms (Yahel et al. 2003). DOC

consists of nutrients and chemicals dissolved in the water (Ribes et al. 1999). Sponges preferentially feed on POC (McMurray et al. 2016), but the bulk of a sponge's diet is comprised of DOC (Ribes et al. 1999, Yahel et al. 2003) due to the much higher abundance of DOC in ocean waters (Hansell & Carlson 2002, Kaiser & Benner 2009). Some sponge species also receive supplemental nutrition via photosynthesis from symbiotic cyanobacteria that live in sponge tissue (Erwin & Thacker 2008). The carbon matter that is filtered by the sponge is phagocytized and digested inside cells, where the nutrients can then be used for the sponge's energetic needs (Wehrl & Hentschel 2007).

Sponge Secondary Metabolites

In addition to the chemicals that sponges use for basic life processes, called primary metabolites, sponges produce a suite of chemicals that are used for other purposes, termed secondary metabolites (Pawlik 2011). These secondary metabolites are used as an alternative to physical defenses, as many sponges are relatively soft-bodied and cannot move to defend themselves. Some uses of secondary metabolites include deterring predation by fish and other spongivores, preventing fouling of sponge surfaces, inhibiting growth from nearby competing species, and acting as antimicrobial defenses for the sponge (Pawlik 1993, Kelly et al. 2005, Slattery & Gochfeld 2012, Gochfeld et al. 2012, Puyana et al. 2015, Slattery et al. 2016). These metabolites are often chemically expensive to make, as they are usually complex compounds or present in high concentrations (Pawlik 1993), so sponges must trade off the energy used to produce metabolites with the benefits they provide. For example, sponges that produce compounds that inhibit predation are able to save energy by not having to regrow tissue that has been eaten (Cronin 2001). Of particular interest to researchers are the metabolites

that perform antibacterial roles in sponge defense. These antimicrobial chemicals are used to prevent colonization by potentially pathogenic bacteria and provide protection from diseases they may cause (Gochfeld & Aeby 2008, Gochfeld et al. 2012). In addition to providing protection for the sponges, researchers can isolate chemicals with antimicrobial and other properties and study them for possible development as drugs for humans (Kelman et al. 2009, Newman & Cragg 2014).

While many sponge species produce secondary metabolites, the chemicals that are produced and their effects are very different based on the needs of the sponge (Kelman et al. 2009, Loh & Pawlik 2014). Differences in secondary metabolites can also be found within the same species of sponge. For example, some sponges may produce antimicrobial chemicals only when diseased, and may not need these chemicals when the sponge is otherwise healthy (Gochfeld & Aeby 2008, Gochfeld et al. 2012). A sponge's location can also affect the chemicals a sponge species will produce due to local biotic and abiotic factors that vary geographically (Rohde et al. 2012). Abiotic factors can include pollution and runoff that reach reefs near shores, water temperature, and exposure to UV light (Rohde et al. 2012, Loh & Pawlik 2014). Biotic factors can include the life cycle of the sponge, predation, invasion by competing organisms, and attack by bacteria and other microbes (Rohde et al. 2012, Slattery & Gochfeld 2012, Loh & Pawlik 2014).

Xestospongia muta

The sponge *Xestospongia muta* is a giant barrel sponge found in many reef communities across the Caribbean basin (McMurray et al. 2008). *Xestospongia muta* individuals can grow to sizes of over 1 meter across the mouth of the barrel (McMurray et al. 2008) and can potentially live to over 100 years of age, earning them the nickname

"Redwood of the reef" (Gammill 1997, McMurray et al. 2008). *Xestospongia muta* is abundant across a broad range of depths, ranging from shallow (e.g., <30m in depth), to mesophotic (e.g., 30-150m in depth; Lesser et al. 2009) reefs (McMurray et al. 2008, Morrow et al. 2016). *Xestospongia muta* is classified as a high microbial abundance sponge, meaning it contains between 10^8-10^{10} bacteria per gram of sponge wet weight (Hentschel et al. 2006). Within this large bacterial community, there are many phyla represented, including Cyanobacteria, which allows for photosynthesis in *X. muta* and contributes to its coloration (Fiore et al. 2013, Olson & Gao 2013), as well as Proteobacteria, Chloroflexi, and Actinobacteria, among others (Erwin & Thacker 2008, Fiore et al. 2013, Villegas-Plazas et al. 2018).

Xestospongia muta is susceptible to sponge orange band disease (SOB), a fatal bleaching event with an orange band of color at the boundary of healthy and bleached tissue (Cowart et al. 2006, Angermeier et al. 2011). This disease can lead to total bleaching within 6 weeks, and although attempts were made to identify a causative pathogen, there was no evidence of specific microbial involvement, so no etiologic agent has been determined (Cowart et al. 2006, Angermeier et al. 2011). While *X. muta* may not exhibit resistance to SOB, it produces over 40 secondary metabolites, including multiple brominated acetylenic acids, multiple ene-yne tetrahydrofurans, xestosterol, mutasterol, brominated hydrocarbons, and long chain polyacetylenes (Schmitz & Gopichand 1978, Morinaka et al. 2007, Zhou et al. 2011, Ankisetty & Slattery 2012). These compounds extracted from *X. muta* have been found to have various biological effects, including antimicrobial activity against *Pseudomonas aeruginosa, Mycobacterium intracellulare* (Ankisetty & Slattery 2012), and *Cryptococcus neoformans* (Morinaka et al. 2007), antitumor properties (Agustina et al. 2018),

antimalarial properties (Rakotondraibe 2014), inhibiting feeding by parrotfish and hermit crabs (Dunlap & Pawlik 1998), and inhibition of HIV protease (Patil et al. 1992).

This Study

It has been shown that production and content of secondary metabolites can vary within a sponge species due to various factors associated with temperature variability (Sacristán-Soriano et al. 2012), size of the organism (Luter & Duckworth 2010), and in response to various stressors (Gochfeld et al. 2012, Slattery & Gochfeld 2012, Slattery et al. 2016, Lesser et al. 2016), but there has been limited study into variation across large geographic areas (Rohde et al. 2012). *Xestospongia muta* is a widespread species in the Caribbean, yet variability in its production of chemical defenses remains unknown.

The purpose of this study was to examine potential geographic variation of antibacterial activity in the marine sponge *X. muta* at four locations across the broader Caribbean basin: Curaçao, Belize, Grand Cayman, and St. Croix, US Virgin Islands (USVI). Potential variation in chemical defenses was determined by testing the antimicrobial properties of *X. muta* against selected human and marine bacterial pathogens and comparing rates of bacterial growth when exposed to sponge extracts from the different regions.

Methods

Sample Collection

Pieces (approximately 100 cm^3) of 5-10 replicate *X. muta* sponges (Figure 1) were collected by hand by divers using SCUBA at a depth of 15 m from 2-3 sites in each of 4 countries (Figure 2): Curaçao (Pescadero Bay, Double Reef), Belize (Southwater Cay, Carrie Bow Cay, Curlew Cay), Grand Cayman (Sentinel Rock, Kittiwake Anchor Buoy, Coconut Bay Mini-wall), and St. Croix, USVI (Cane Bay, Eagle Ray, Salt River). The sponge samples were collected into individual plastic bags and frozen prior to transport to the University of Mississippi for analysis.

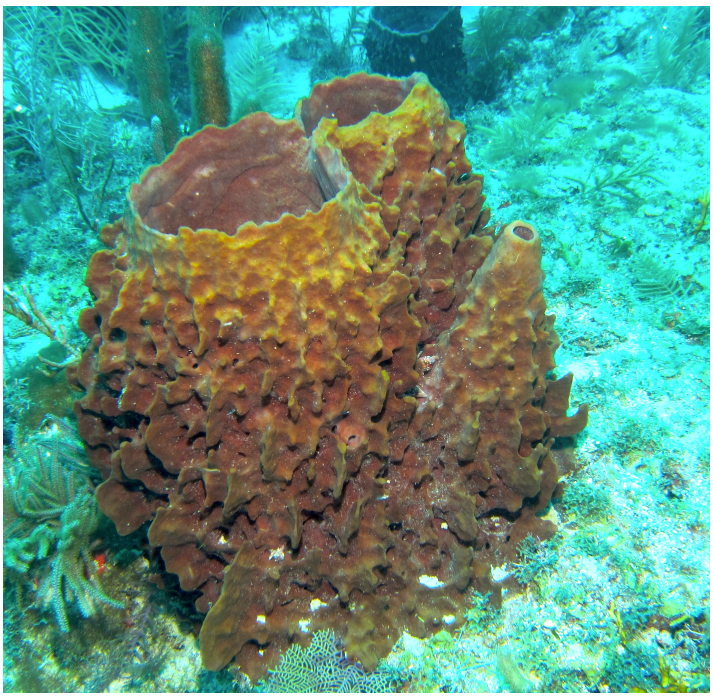

Figure 1. Two adult *Xestospongia muta*, with a juvenile sponge budding off to the side. Photo courtesy of Deborah Gochfeld.

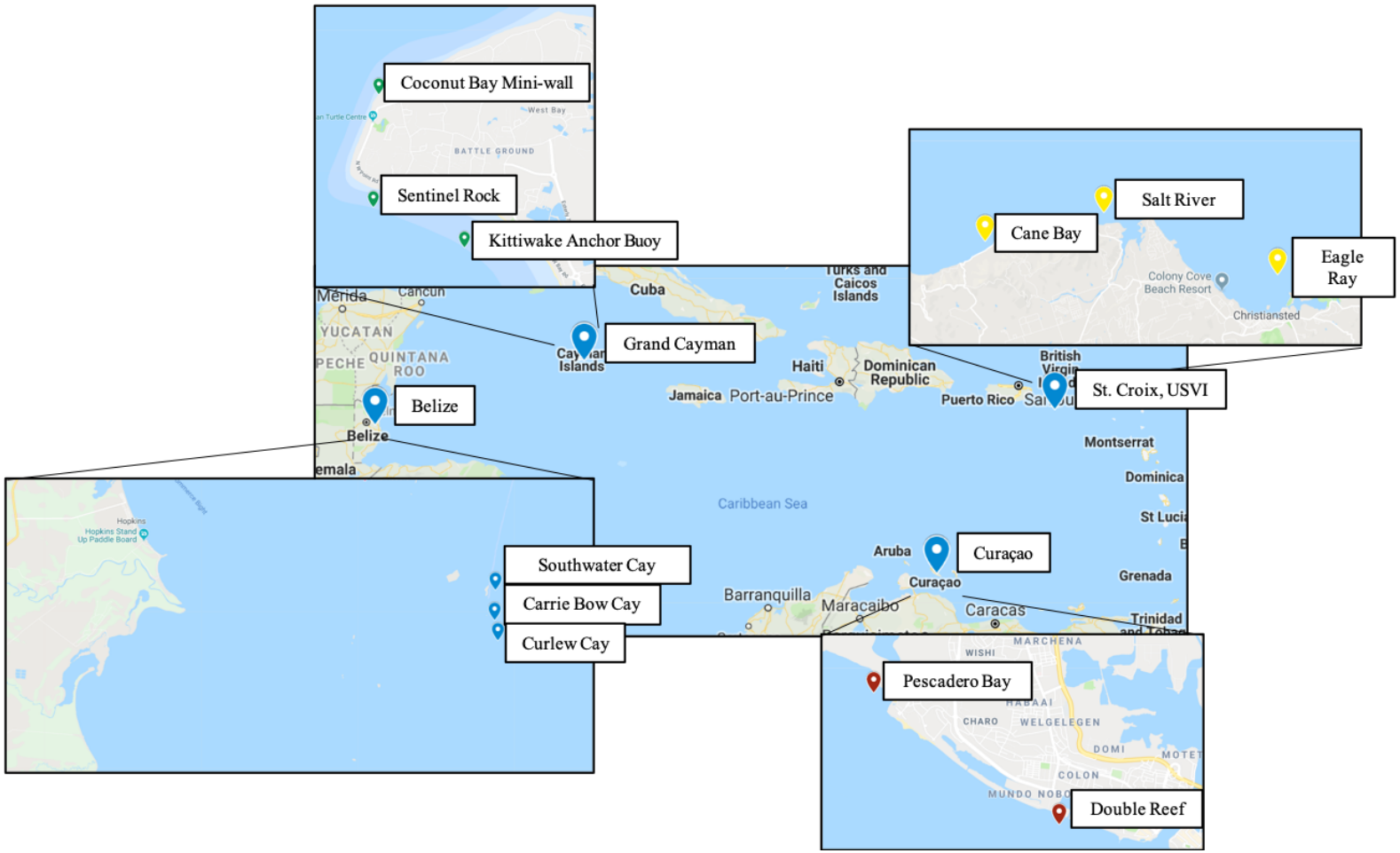

Figure 2. A map of the Caribbean showing the four countries from which sponges were collected. Insets show the specific collection sites within each country.

Sample Preparation

A small (approximately 30 cm^3) piece of sponge was cut from each sample. The wet mass and volume of the sponge piece were measured and recorded so that sponge tissue weight-to-volume ratios could be calculated. Each sponge piece was then placed into an individual labeled bag and frozen. Once fully frozen, the sponge samples were lyophilized until completely dry. The dried samples were weighed and recorded to obtain a wet-to-dry weight ratio for future calculations. Each sample was crushed to produce a powder for chemical extraction.

Sample Extraction

For sample extraction, 300 mg of the sponge powder from each sample was measured into a pre-weighed and labeled 8-dram glass vial. The powder was extracted with 10 mL of 1:1 dichloromethane:methanol, and sonicated for 15 minutes in a Branson 5510 sonicator. The vials were left to settle overnight, and the supernatant was pipetted into a clean, pre-weighed and labeled 8-dram vial, after which the extracts were evaporated using a Savant Speedvac Plus. This process was repeated twice. The three extracts were combined into one vial, dried in the speedvac, and a final dry mass of the extract was obtained. The mass of each sponge extract was divided by the volume of the sponge sample extracted, and these numbers were averaged across all sponges to obtain a natural concentration of the sponge extract (mg/mL). The average natural concentration was found to be 35.5 mg/mL. The extracts were dissolved in appropriate amounts of dimethyl sulfoxide (DMSO) to bring the concentration to 100 mg/mL which, when diluted by 1/20 in the antibacterial assay, would provide test concentrations of $1/7^{th}$ that of natural concentrations of the sponge extracts.

Antibacterial Assays

The antibacterial assay methods were adapted from those used in Gochfeld and Aeby (2008), and follow the assay used in Vickers (2017). Each of the sponge extracts was tested using four bacterial strains: *Serratia marcescens, Aurantimonas coralicida, Vibrio coralliilyticus,* and *Yersinia enterocolitica. Vibrio coralliilyticus*, associated with bleaching of the coral *Pocillopora damicornis* (Ben-Haim et al. 2003), and *Aurantimonas coralicida,* the causative agent of white plague in the coral *Dichocoenia stokesi* (Denner et al. 2003), are coral specific pathogens. *Serratia marcescens* is a human enterobacteria associated with white pox disease of the elkhorn coral *Acropora palmata* (Patterson et al. 2002). These coral pathogens were selected in lieu of sponge-specific pathogens, as none have been identified to date, except for a proteobacterium that causes sponge boring necrosis in the Australian sponge *Rhopaloeides odorabile* (Webster et al. 2002). *Yersinia enterocolitica* is also a human enterobacterium that can survive in the ocean and could potentially affect sponges (Gochfeld & Aeby 2008). The bacterial strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DMSZ, Braunschweig, Germany).

Bacteria were grown from frozen stock solutions by pipetting 10 mL of an appropriate growth medium for each bacterial strain into an autoclaved 50 mL Erlenmeyer flask. *Vibrio coralliilyticus* and *A. coralicida* were grown in marine broth while *S. marcescens* was grown in trypticase soy broth (Patterson et al. 2002, Gochfeld & Aeby 2008) and *Y. enterocolitica* was grown in tryptose media (Weagant & Kaysner 1983, Gochfeld & Aeby 2008). The flask of growth media was inoculated with 25 µL of a frozen stock of the bacteria, and the culture was placed into a rocking incubator at the

optimal growth temperature for each bacterium. *Yersinia enterocolitica* was grown at 37°C, and the other three bacteria were grown at 28°C (Gochfeld & Aeby 2008, Vickers 2017). Cultures were incubated for 24 h before plating.

After 24 h, 1 mL of culture was pipetted into a sterile microcentrifuge tube and centrifuged at 13,200 rpm for 5 min to form a pellet. The supernatant was removed and replaced with 1 mL of fresh media, and the bacteria were resuspended by pipetting the mixture and then vortexing. The bacterial culture (100 µL) was pipetted into a second sterile microcentrifuge tube, along with 900 µL of media, to produce a 1:10 dilution of the original bacterial culture. The optical density at 600 nm (OD600) for this diluted culture was measured using an Eppendorf BioPhotometer and the value recorded. Calculations were performed to determine the ratio of bacterial culture and media solution needed to create a bacterial stock solution at 0.100 ± 0.01 OD600. Approximately 10 mL of the prepared bacterial stock solution was needed per 96 well plate, and each plate supported 10 sponge samples. When the bacterial stock solution was ready, the sponge extracts dissolved in DMSO were removed from the refrigerator to warm up to room temperature.

The plates were set up following the design in Figure 3. All solutions were run in triplicate except for "media + extract", which were run in duplicate. Wells labeled "media" and "bugs" contained 200 µL of sterile media or the bacterial stock solution, respectively. Wells labeled "cipro" contained 5 µL of a 1 mg/mL solution of ciprofloxacin and were filled with 195 µL of the bacterial stock solution. Ciprofloxacin is a fluoroquinolone antibiotic agent that inhibits growth of the bacteria used in the assay (Huang et al. 1999, Jayahar et al. 2002). It serves as a positive control for comparison of the strength of any antibiotic effect of the sponge extracts. In each well labeled "extract",

10 µL of the corresponding sponge extract was added to 190 µL of the bacterial stock solution. Wells labeled “media + extract” contained 10 µL of the same corresponding extract added to 190 µL of the sterile media. The wells containing “media” and “media + extract” are included to control for the color of the media and sponge extracts, respectively, and the wells of pure bacterial stock solution serve as a negative control of uninhibited bacterial growth (Gochfeld & Aeby 2008, Vickers 2017). After plating, the OD600 of the entire plate was read using a BioTek Synergy 2 plate reader. These initial absorbance values were saved into a spreadsheet, and the plate was returned to the rocking incubator and maintained under culture conditions for 24 h. After the 24 h growth period, the plate was removed from the incubator and OD600 was measured again. These final values were saved into a spreadsheet for calculations of bacterial growth. The plate was then disposed of into an appropriate biohazard waste container.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Media	Media	Media		Media	Media	Media					
B	Bugs	Bugs	Bugs		Bugs	Bugs	Bugs					
C	Cipro	Cipro	Cipro		Cipro	Cipro	Cipro					
D	Extract 1	Extract 1	Extract 1		Extract 6	Extract 6	Extract 6		Media + Extract 1	Media + Extract 1	Media + Extract 6	Media + Extract 6
E	Extract 2	Extract 2	Extract 2		Extract 7	Extract 7	Extract 7		Media + Extract 2	Media + Extract 2	Media + Extract 7	Media + Extract 7
F	Extract 3	Extract 3	Extract 3		Extract 8	Extract 8	Extract 8		Media + Extract 3	Media + Extract 3	Media + Extract 8	Media + Extract 8
G	Extract 4	Extract 4	Extract 4		Extract 9	Extract 9	Extract 9		Media + Extract 4	Media + Extract 4	Media + Extract 9	Media + Extract 9
H	Extract 5	Extract 5	Extract 5		Extract 10	Extract 10	Extract 10		Media + Extract 5	Media + Extract 5	Media + Extract 10	Media + Extract 10

Figure 3. Representative layout for an antibacterial assay performed in a 96-well plate. Each cell represents a well and the label indicates its contents (described in detail in the text). Blank cells represent empty wells.

Data Processing

The triplicate or duplicate values for each set of wells were averaged to obtain mean initial and final OD600 values. For each plate, the mean OD600 value of the

"media" or "media + extract" wells at each time point was subtracted from the mean OD600 value of the untreated bacteria ("bugs") or bacteria treated with the corresponding sponge extract ("extract") at that time point, respectively. Potential differences in bacterial growth between plates were normalized by subtracting the mean OD600 values of the untreated bacteria from those treated with extract on the same plate. For all sponge extracts, slopes approximating a bacterial growth curve were then obtained by subtracting the mean OD600 values at 24 h from those at 0 h. A positive slope indicated that the sponge extract promoted bacterial growth, whereas a negative slope indicated that the sponge extract inhibited bacterial growth. The slope values for bacterial growth across the sites and for each bacterial strain were then used in statistical analyses.

Data Analysis

A one-sample t-test was conducted on the set of slope values from each site for each bacterial strain to determine whether the sponge extracts significantly affected bacterial growth. A one-way Analysis of Variance (ANOVA) was performed for each country to test whether, for each bacterial strain, the slope values across the 2-3 sites within a country differed from each other. Since there were minimal differences among sites within countries, all slope values within a country were combined, and one-way ANOVAs were used to test whether the extracts of sponges from the different countries differed in their effects on the growth of each bacterial strain. For ANOVAs that were significant ($p < 0.05$), a post-hoc Tukey's Honestly Significant Difference test was performed to see which sites were significantly different from each other.

Results

Site Results

Average slope values of the bacterial growth curves by site are graphed for each bacterial strain in Figure 4. In general, the antibacterial activity of the sponges was similar among sites within each country but varied among countries and pathogens. For *V. coralliilyticus,* only the sponges from Belize exhibited significant inhibitory activity (t-tests, $p < 0.0005$; Figure 4A). The level of bioactivity against *V. coralliilyticus* in the other countries varied, with the sponges from St. Croix showing a mild, but statistically insignificant (t-tests, $p > 0.05$), stimulating effect. For *V. coralliilyticus,* there was no significant difference between sites within any of the countries (ANOVA, $p > 0.05$). All sites showed significant inhibition of *A. coralicida* growth (t-tests, $p < 0.05$), with an overall similar level of inhibition between sites (Figure 4B), with one exception. There was variability between sites in antibacterial activity of sponges from St. Croix against *A. coralicida* (ANOVA, $p = 0.0044$), with significantly less growth inhibition by Eagle Ray sponges (Tukey's HSD, $p = 0.003$) than by Salt River sponges, although only mildly less inhibition by Eagle Ray than by Cane Bay sponges (Tukey's HSD, $p = 0.069$). Sponges from all sites in three countries, Belize, Curaçao, and Grand Cayman, exhibited significant inhibition against *S. marcescens* (t-tests, $p < 0.05$; Figure 4C), whereas there was no effect on bacterial growth by sponges from St. Croix. The effects of sponge extracts on *Y. enterocolitica* growth curves were highly variable (Figure 4D), with only three sites exhibiting significant inhibition (t-tests, $p < 0.05$), Curlew Cay in Belize,

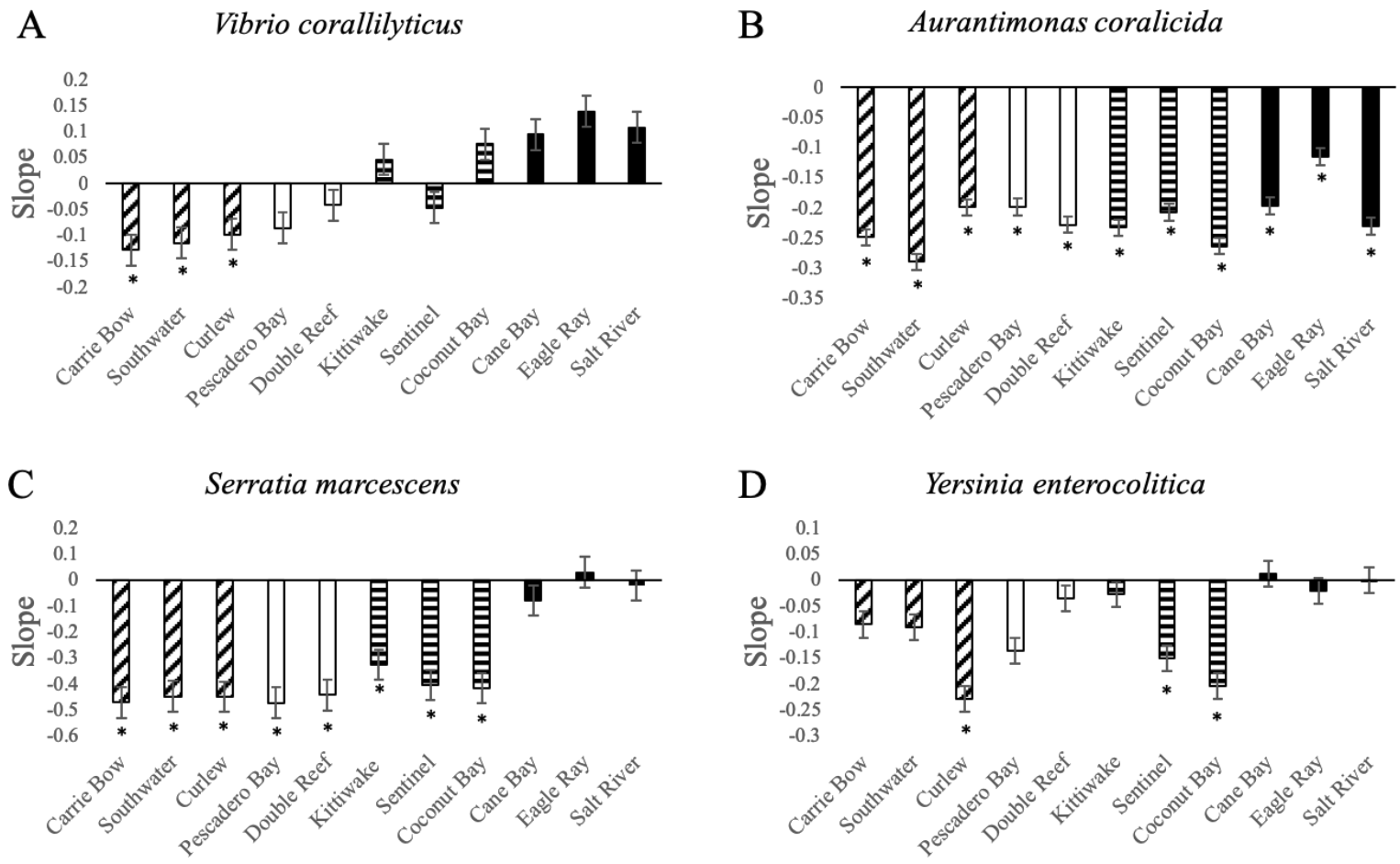

Figure 4. Slopes of the bacterial growth curves (mean ± SE, n = 5-10 sponges) for 4 bacterial strains exposed to extracts from *Xestospongia muta* from the labeled sites in four countries: Belize (diagonal striped bars), Curaçao (white bars), Grand Cayman (horizontal striped bars), and St. Croix (solid bars). Greater negative values indicate more inhibitory extracts. *Slope is significantly different from 0 at $p < 0.05$ (one sample t-tests). Note that each graph has a different scale.

and Sentinel Rock and Coconut Bay Mini-wall in Grand Cayman. Most other sites showed minimal activity or mild inhibition.

Country Results

Figure 5 shows the mean slopes of the growth curves for all of the sites combined within each country for each bacterial strain. For *V. coralliilyticus*, there was a significant difference in antibacterial activity among countries (ANOVA, $p < 0.0001$; Figure 5A). The bacterial growth of *V. coralliilyticus* exposed to extracts from the sponges from Belize was significantly lower (Tukey's HSD, $p < 0.05$) than from the other three countries. Sponge extracts from Curaçao exhibited significantly greater inhibition against *V. coralliilyticus* than did extracts from St. Croix (Tukey's HSD, $p < 0.05$), but neither differed significantly from sponge extracts from Grand Cayman (Tukey's HSD, $p > 0.05$). Sponges from all of the countries exhibited similar levels of growth inhibition against *A. coralicida* (ANOVA, $p = 0.13$; Figure 5B). For *S. marcescens,* there was a significant difference in bacterial growth between countries (ANOVA, $p < 0.05$; Figure 5C). Sponges from Belize, Curaçao, and Grand Cayman had similar effects on bacterial growth, but St. Croix sponges were significantly less inhibitory than sponges from the other three countries (Tukey's HSD, $p < 0.05$). Visually, it appears as if *Y. enterocolitica* would exhibit differences between countries in the antibacterial effect of the sponges, but the observed differences were not statistically significant (ANOVA, $p = 0.107$; Figure 5D).

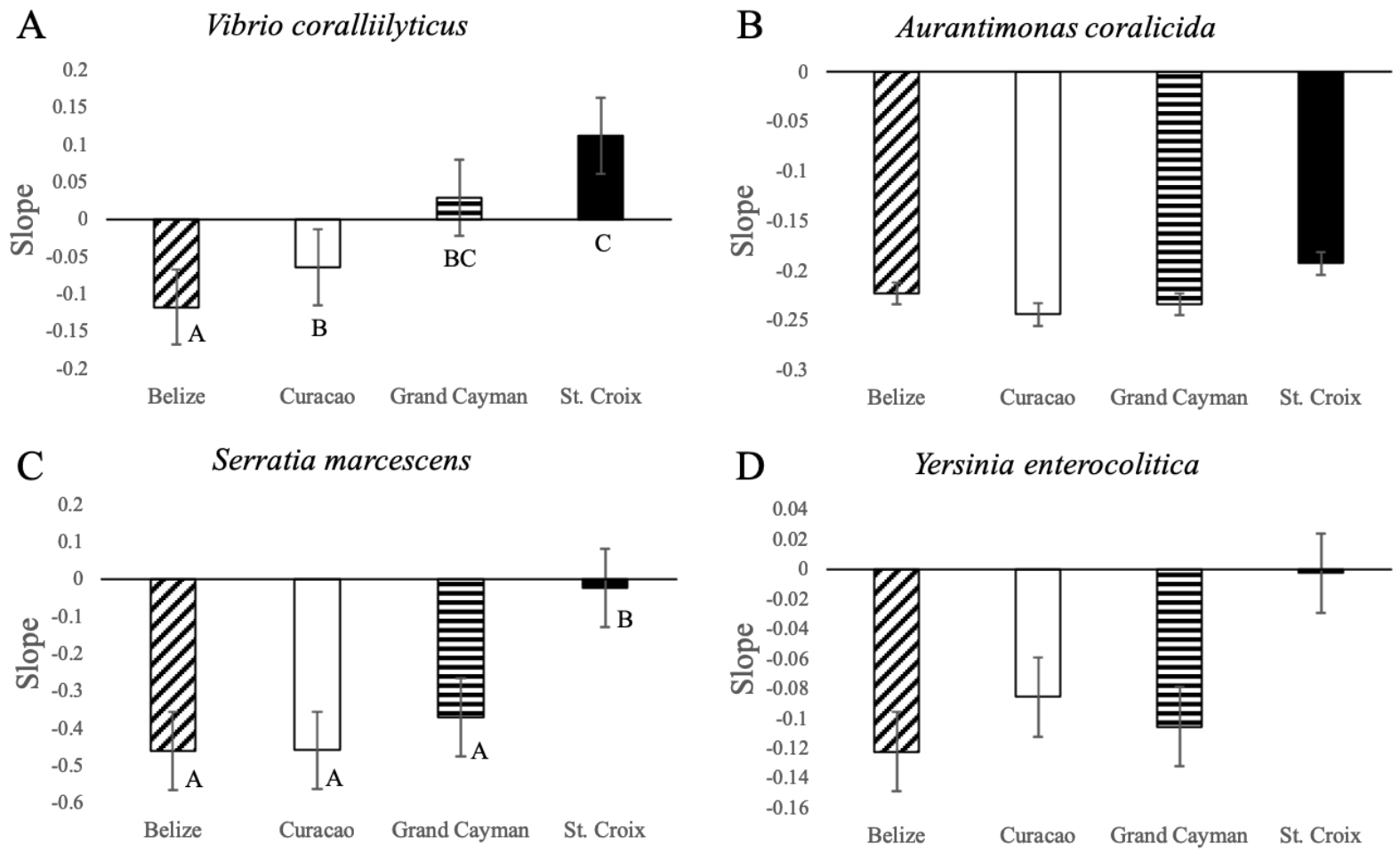

Figure 5. Slopes of the bacterial growth curves (mean ± SE, n = 10-20 sponges) for 4 bacterial strains exposed to extracts from *Xestospongia muta* from the labeled countries. Greater negative values indicate more inhibitory extracts. Letters by the bars indicate countries that are significantly different from each other at $p < 0.05$ (Tukey's HSD). Note that each graph has a different scale.

Discussion

Geographic Variability in Chemical Defenses

This study found variable antibacterial activity of *X. muta* extracts against a panel of bacterial strains selected to represent potential marine pathogens. The patterns of inhibition, or lack thereof, indicate that the chemical defenses have specific, as opposed to broad-spectrum, activity. Selectivity allows sponges to expend energy effectively in responding to geographically variant stressors. Sponges need to expend energy to protect against the greatest threats and may not need to defend against all kinds of pathogens.

There were not many differences in the antibacterial activity between sites within a country, which is not surprising due to the relative proximity of the collection areas. One country that did show differences was St. Croix, in which the sponge extracts varied in their activity against *A. coralicida* (Figure 4B). The extracts of sponges from Eagle Ray inhibited bacterial growth significantly less than those from Salt River, indicating that there is a major difference in the area. Due to its location outside of a main harbor, Eagle Ray is significantly more impacted than the other sites on St. Croix (Gochfeld, personal communications), and received approximately half of the light intensity than those other sites during a measured time period (Slattery & Gochfeld, unpublished data). *Xestospongia muta* have cyanobacteria that photosynthesize and contribute to the overall energy of the sponge (Erwin & Thacker 2007, Fiore et al. 2013). The reduced light intensity could reduce the amount of photosynthesis, which would provide the sponges

with less energy. This reduced supply of energy to sponges from Eagle Ray might affect their ability to produce secondary metabolites, which could result in the lower levels of antibacterial activity seen in this study.

Within the ocean, different geographic regions provide many different environmental conditions that will affect how a sponge balances secondary metabolite production with other energetic demands, and some factors may contribute more heavily in certain areas than in others. With broad geographic variation, there can be major differences in factors such as temperature, nutrient levels, and amount of predation on sponges. Variation in external factors may not be the only explanation, however, for altered antibacterial activity. Intrinsic factors such as a sponge's genome or the bacteria that make up its microbiome can also affect what chemicals are produced, and influence both local and broad-scale differences.

One non-microbial stressor that can variably affect sponges is predation. A study done on Caribbean sponge chemical defenses against predation (Loh & Pawlik 2014) compared predator abundance and the diversity of the sponges present at many sites across the Caribbean to determine how feeding pressure selected for sponge species. *Xestospongia muta* is variably chemically defended (Pawlik et al. 1995, Loh & Pawlik 2014), which suggests potential impacts due to the abundance of spongivores. Locations that are marine protected areas do not have fishing pressure, which leads to high abundances of predators that can eat sponges, which creates selective pressure for more defended sponges. In these locations, *X. muta* and other sponges would have to expend more energy to deter predation. The sites used in my study vary in their levels of fishing pressure (Gochfeld & Slattery, personal communication), so secondary metabolite

variation in these sponges could be caused by differences in predation pressure, but that is difficult to separate from other environmental factors.

There have been studies in other sponges that have examined potential geographic variance in antibacterial activity (Rohde et al. 2012, Vickers 2017). Rohde et al. (2012) studied the Indo-Pacific sponge *Stylissa massa* and how its production of secondary metabolites changed over small scales, such as among sites and depths within Guam, and large scales, across the Pacific Ocean. They found that there was variation in secondary metabolite production over the large scales, as in my study, but they also found that metabolite levels showed greater variation between local sites than across large scales. Their data did not point to any particular cause for the local or broad scale variation, as there were few and many differences, respectively, in biotic and abiotic factors (Rohde et al. 2012). Vickers (2017) studied the antibacterial activity and chemical composition of sponges from three *Aplysina* morphotypes across various locations in the Caribbean. It was found that, within morphotype and health status, the antibacterial activity and chemical profiles varied between the countries, supporting the results of my study. Stockton (2016) studied chemical variability in the same *Aplysina* morphotypes from St. Thomas, USVI and the Bahamas by examining chemical profiles and antibacterial activity of the sponges using the same bacterial panel as in Vickers (2017) and my study. All of the *Aplysina* morphotypes showed varying levels of inhibition against *V. coralliilyticus, A. coralicida,* and *S. marcescens*, but significantly stimulated growth of *Y. enterocolitica*. The profiles of inhibition varied and again support specificity of antibacterial defenses. Both Stockton (2016) and Vickers (2017) suggested that anthropogenic factors (e.g., sedimentation and nutrient run-off), contributed to the

geographic variation in secondary metabolites in addition to other potential biotic and abiotic factors.

Antibacterial Chemical Defenses

Sponges are exposed to many bacteria in the surrounding seawater, but still generally produce specific antibacterial chemicals as opposed to broad-spectrum defenses. If sponges were to produce multiple broad-spectrum chemicals, they could negatively affect the bacterial symbionts that live within the sponges and contribute to important physiological processes for the sponge, including the production of secondary metabolites. Producing chemical defenses with specific targets avoids affecting the microbiome of the sponge and can allow for induced response to a particular stressor over continuous production (Teeyapant & Proksch 1993, Hill & Hill 2002, Gochfeld 2004). Inducible responses allow for an organism to chemically respond to a stressor when needed, but not waste energy to constitutively express the defenses if the stressor is an uncommon occurrence (Hill & Hill 2002).

While *X. muta* does not contain compounds with broad-spectrum antibiotic effects, it does produce a number of compounds with antimicrobial activity, and would be expected to show mild effects on bacterial growth of many different strains. Newbold et al. (1999) tested the antibacterial effects of various sponge species from the Florida coastline, including *X. muta.* They tested against 8 marine bacterial strains and found that *X. muta* inhibited growth of two of the bacteria. My study showed at least some activity against all of the bacterial strains tested, with different levels of effectiveness. Against three of the bacterial strains, there were either inhibitory effects or no effects on growth. The growth pattern of *V. coralliilyticus* (Figure 4A) was interesting, as sponges from

Belize inhibited bacterial growth, but those from some sites in Grand Cayman and St. Croix appeared to stimulate bacterial growth. The secondary metabolites that sponges produce are organic compounds, and thus a carbon source, which bacteria can use as a nutrient source (Kritzberg et al. 2004, Pozuelo et al. 2012) if there are not active compounds to inhibit growth. This is likely why there was mild growth stimulation of *V. coralliilyticus* by the sponge extracts from Grand Cayman and St. Croix, and indicates that the sponges from these two countries may be missing one or more active compounds that extracts from the other two countries possess.

There have been multiple studies that tested sponge antibacterial activity against the bacterial strains used in this study (Gochfeld et al. 2012, Stockton 2016, Vickers 2017). These studies were on different morphotypes of *Aplysina*, and these sponges exhibited varying levels of inhibitory effects on *V. coralliilyticus, A. coralicida,* and *S. marcescens*. Some of the assays showed stimulation of growth for *Y. enterocolitica* but others had minimal activity against *Y. enterocolitica*. These activity patterns are different from those of *X. muta*, but that is to be expected, as the sponge species produce completely different types of secondary metabolites (Gochfeld et al. 2012, Ankisetty & Slattery 2012, Villegas-Plazas et al. 2018). The antibacterial activity of *Aplysina* spp. did align with the specificity of *X. muta,* as neither showed broad-spectrum activity and the levels of inhibition were specific to each bacterial strain. In all of the studies with these bacterial strains, the microbes were chosen as proxies for potential sponge pathogens. The antibacterial activity exhibited against these strains is likely representative of what the activity would be against sponge pathogens, but could differ if sponge-specific pathogens exhibit significantly different methods of action from coral and human

pathogens in the same way that results from studies on model organisms do not directly translate into functions in humans.

Areas for Further Study

This study investigated whether there were differences in antibacterial activity of sponge extracts due to geographic variation, but did not directly study the chemical profiles of these sponges. Analysis of the chemical profiles by High Performance Liquid Chromatography (HPLC) or other methods could indicate whether the variation in antibacterial activity is caused by altered quantities of metabolites produced or if there are different chemicals being produced by the sponges from different locations. To date, one disease, sponge orange band, has been described from *X. muta,* and it has not been shown to have an etiologic pathogen (Angermeier et al. 2011), but *X. muta* may still exhibit a change in chemical defense production in response to disease or other stressors. Further study is needed into the causative agents of this disease and any chemical responses that may be exhibited by *X. muta* and other sponge species that are affected by diseases and other stressors.

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